

RESEARCH OUTPUTS / RÉSULTATS DE RECHERCHE

Methyl- β -cyclodextrin concurs with interleukin (IL)-4, IL-13 and IL-25 to induce alterations reminiscent of atopic dermatitis in reconstructed human epidermis

De Vuyst, Evelyne; Giltaire, Séverine; Lambert de Rouvroit, Catherine; Malaisse, Jérémy; Mound, Abdallah; Bourtembourg, Maureen; Nikkels, Arjen F; Chrétien, Aline; Salmon, Michel; Poumay, Yves

Published in:
Experimental dermatology

DOI:
[10.1111/exd.13113](https://doi.org/10.1111/exd.13113)

Publication date:
2018

Document Version
Peer reviewed version

[Link to publication](#)

Citation for pulished version (HARVARD):

De Vuyst, E, Giltaire, S, Lambert de Rouvroit, C, Malaisse, J, Mound, A, Bourtembourg, M, Nikkels, AF, Chrétien, A, Salmon, M & Poumay, Y 2018, 'Methyl- β -cyclodextrin concurs with interleukin (IL)-4, IL-13 and IL-25 to induce alterations reminiscent of atopic dermatitis in reconstructed human epidermis', *Experimental dermatology*, vol. 27, no. 4, pp. 435-437. <https://doi.org/10.1111/exd.13113>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Received Date : 23-Mar-2016

Revised Date : 20-May-2016

Accepted Date : 03-Jun-2016

Article type : Letter to the Editors

M β CD concurs with IL-4, IL-13 and IL-25 to induce alterations reminiscent of atopic dermatitis in reconstructed human epidermis

Evelyne De Vuyst¹, Séverine Giltaine¹, Catherine Lambert de Rouvroit¹, Jérémy Malaisse¹, Abdallah Mound¹, Maureen Bourtembourg¹, Arjen F. Nikkels², Aline Chrétien³, Michel Salmon³ and Yves Poumay¹

1 URPhyM-NARILIS, University of Namur, Namur, Belgium.

2 Department of Dermatology, University Medical Center of Liège, Liège, Belgium.

3 StratiCell, Les Isnes, Belgium.

Correspondence: Professor Yves Poumay, Cell and Tissue Laboratory, URPhyM-NARILIS, University of Namur, 61, rue de Bruxelles, B-5000 Namur, Belgium.

E-mail: yves.poumay@unamur.be

Phone: +32 81 72 42 57

Keywords : reconstructed human epidermis, atopic dermatitis, interleukins, epidermal barrier and methyl- β -cyclodextrin.

Abbreviations: AD, atopic dermatitis; TSLP, thymic stromal lymphopoietin; RHE, reconstructed human epidermis; M β CD, methyl- β -cyclodextrin; IL, interleukin; TEER, trans-epidermal electrical resistance; FLG, filaggrin; LOR, loricrin; CA2, carbonic anhydrase II; NELL2, neural epidermal growth factor-like 2 ; HAS, hyaluronic acid synthase; HA, hyaluronic acid.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/exd.13113

This article is protected by copyright. All rights reserved.

BACKGROUND

Reconstructed human epidermis (RHE) mimic normal human *in vivo* epidermis in terms of histology, distribution of differentiation markers, and barrier functionality (1).

A typical transcriptional profile and the activation of signalling pathways reminiscent of atopic dermatitis (AD) lesional skin can be obtained in RHE upon incubation with methyl- β -cyclodextrin (M β CD) (2, 3), a molecule that extracts cholesterol from plasma membranes, thereby disrupting lipid microdomains. However, barrier function and morphology remain unaltered in those conditions, requiring further refinement of the model.

QUESTION ADDRESSED

Because of the crucial role played by Th2 immune response in AD, a mixture of interleukins linked to this Th2 response (IL-4, IL-13 and IL-25) was used in addition to M β CD, in an attempt to induce most of the epidermal AD features in RHE. Ultimately, a valid RHE model of acute AD would allow studying the epidermal component of pathogenesis.

EXPERIMENTAL DESIGN

RHE were incubated for two hours with M β CD in order to induce cholesterol depletion (Figure S1), then for 48 hours with the interleukin mix and compared to control tissues, and to tissues incubated with either M β CD or interleukins.

RESULTS

When allowed to recover in fresh culture medium after incubation with M β CD for 2 hours, RHE revealed no obvious histological alteration when compared with untreated RHE (Figure 1a). Conversely, RHE treated with IL-4, IL-13 and IL-25 for 48 hours displayed intercellular space widening similar to spongiosis (already reported for IL-4 and IL-13 (4-6)) and hypogranulosis, two histological hallmarks of lesional AD skin (Figure S2). These morphological alterations were enhanced when keratinocytes were challenged by M β CD before being incubated with the three interleukins (Figure 1a).

Barrier function, weakened in AD, was studied using two assays: measurement of trans-epithelial electrical resistance (TEER) and assessment of permeability to the fluorescent dye lucifer yellow (LY) through the RHE. A significant decrease in TEER was observed in RHE incubated with the interleukins, and worsened upon membrane cholesterol depletion (Figure 1b). Accordingly, permeability of RHE towards LY was significantly increased after combined treatments, whereas cholesterol depletion alone or incubation with interleukins only were insufficient to elicit an effect (Figure 1c).

Then, expression of atopic dermatitis markers was investigated. Gene expression of filaggrin (FLG) and loricrin (LOR), epidermal differentiation genes usually downregulated in lesional AD, was analyzed through RT-qPCR (Figure 2a). FLG and LOR exhibited reduced mRNA levels in RHE incubated with IL-4, -13 and -25, as already reported in the literature regardless of the different concentrations and timings used (5). This decrease was exacerbated and became significant when keratinocytes were challenged by cholesterol depletion before incubation with the interleukins. Simultaneously, relative expression levels of carbonic anhydrase II (CA2) and neural epidermal growth factor-like 2 (NELL2), two genes upregulated in AD (7), were significantly enhanced in RHE in response to incubation with interleukins (Figure 2a).

Immunohistological analysis confirmed decreased expression of LOR and increased expression of CA2 in AD lesions (Figure S3a). Similar changes were observed in RHE treated with interleukins and became even more evident in tissues previously treated with M β CD (Figure S3b).

Expression levels for hyaluronan synthase 3 (HAS3) were found elevated in RHE exposed to interleukins and further increased after combined treatments (Figure 2a), in agreement with the upregulation observed in AD (4, 8). Though, no significant changes were found with respect to HAS1 expression levels (data not shown), unlike data collected from AD lesions (8).

Fluorescent detection of hyaluronic acid (HA) revealed increased staining of intercellular spaces between keratinocytes in AD lesions (Figure S4a). HA was also more strongly detected in RHE incubated with interleukins, particularly after previous challenging by M β CD (Figure S4b). Accordingly, increased HA concentrations were measured in culture medium of treated RHE (Figure S4c).

TSLP is a cytokine, upregulated in AD, which contributes to Th2 immune response activation, promotes itch in skin and the 'atopic march' in general (9). However, despite significant induction in response to two hours of M β CD (Figure S1c), mRNA expression levels for TSLP were no longer above baseline 48 hours later (Figure 2a). However, in the meantime, over the 6 hours period following cholesterol depletion (Figure 2b), data illustrate that interleukins and M β CD concur to significantly enhance TSLP expression in challenged RHE.

CONCLUSIONS

This study illustrates that challenging RHE through cholesterol depletion, or by incubation with IL-4, IL-13 and IL-25, results in multiple epidermal alterations. But interestingly, a combination of those two treatments has additive effects, allowing mimicking an AD-like epidermal phenotype in vitro in the absence of immune cells. Indeed, morphological alterations such as tissue spongiosis and hypogranulosis, alterations in mRNA expression levels and histological localizations of typical AD and differentiation markers, modulations of epidermal HA synthesis, and epidermal barrier weakening represent hallmarks of AD epidermis.

In vivo, such alterations become responsible for activation of the immune system because they promote penetration of pathogens or allergens, thereby creating some vicious circle likely responsible for AD lesions development (Figure 2c).

Most probably, challenging of keratinocytes by M β CD models alterations in cell signalling through disorganization of specific lipid microdomains in this cell type (3). Interestingly, gene expression levels for IL-13R α 2, IL-13R α 1, IL-4R α and IL-17RA (subunit of IL-25 receptor) were upregulated in keratinocytes incubated with M β CD (3). This indicates that cholesterol-containing lipid microdomains could regulate signalling through these receptors and could therefore explain the additive effects observed in this study.

In conclusion, this study confirms that membrane cholesterol depletion in keratinocytes concurs with Th2-related cytokines to elicit an AD-like phenotype. The present model could be used in order to study other features encountered in AD epidermis, but also evaluate compounds intending to relieve, prevent AD lesions or restore keratinocyte functions.

ACKNOWLEDGEMENTS

We thank B. Balau, V. De Glas, K. De Swert and D. Van Vlaender for technical help. EDV, SG and MB performed the study. EDV, JM and AM analysed the data. YP, AC and MS designed the study. AN contributed the AD biopsies. EDV, CLdR and YP wrote the paper. AC and MS are employees of Straticell. Financial support was provided by the Région Wallonne (BAREPI-convention-1217660) and FRFC 2.4.522.10F grant to YP.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

1. Frankart A, Malaisse J, De Vuyst E, et al. Epidermal morphogenesis during progressive in vitro 3D reconstruction at the air-liquid interface. *Exp Dermatol* 2012; 21: 871-875.
2. De Vuyst E, Giltair S, Lambert de Rouvroit C, et al. The activation of cultured keratinocytes by cholesterol depletion during reconstruction of a human epidermis is reminiscent of monolayer cultures. *Arch Dermatol Res* 2015; 307: 309-318.
3. Mathay C, Pierre M, Pittelkow M R, et al. Transcriptional profiling after lipid raft disruption in keratinocytes identifies critical mediators of atopic dermatitis pathways. *J Invest Dermatol* 2011; 131: 46-58.
4. Ohtani T, Memezawa A, Okuyama R, et al. Increased hyaluronan production and decreased E-cadherin expression by cytokine-stimulated keratinocytes lead to spongiosis formation. *J Invest Dermatol* 2009; 129: 1412-1420.
5. Danso M O, van Drongelen V, Mulder A, et al. TNF-alpha and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *J Invest Dermatol* 2014; 134: 1941-1950.
6. Kamsteeg M, Bergers M, de Boer R, et al. Type 2 helper T-cell cytokines induce morphologic and molecular characteristics of atopic dermatitis in human skin equivalent. *Am J Pathol* 2011; 178: 2091-2099.
7. Kamsteeg M, Jansen P A, van Vlijmen-Willems I M, et al. Molecular diagnostics of psoriasis, atopic dermatitis, allergic contact dermatitis and irritant contact dermatitis. *The British journal of dermatology* 2010; 162: 568-578.
8. Malaisse J, Bourguignon V, De Vuyst E, et al. Hyaluronan metabolism in human keratinocytes and atopic dermatitis skin is driven by a balance of hyaluronan synthases 1 and 3. *J Invest Dermatol* 2014; 134: 2174-2182.
9. Leyva-Castillo J M, Hener P, Jiang H, et al. TSLP produced by keratinocytes promotes allergen sensitization through skin and thereby triggers atopic march in mice. *J Invest Dermatol* 2013; 133: 154-163.

FIGURE LEGENDS

Figure 1. Morphology and barrier efficiency in treated RHE.

(a) Histological sections of RHE treated for 2 hours with M β CD followed by 48 hours of recovery (M β CD 2h + recovery 48h) or treated for 48 hours with IL-4, IL-13 and IL-25 (IL 48h), or a combination of both treatments (M β CD 2h + IL 48h), compared to non-treated RHE (Ctrl 48h). Images are representative of three independent cultures. Scale bar=50 μ m.

Analysis of barrier function, by **(b)** trans-epidermal electrical resistance (TEER) or **(c)** fluorescence quantification of lucifer yellow permeation in the culture medium, of RHE treated for 2 hours with M β CD (M β CD2h), followed or not by 48h incubation with interleukins 4, 13 and 25 (IL 48h). Data represent measurements performed on three independent cultures (error bars = SEM, one-way RM ANOVA and *P<0.05, **P<0.01).

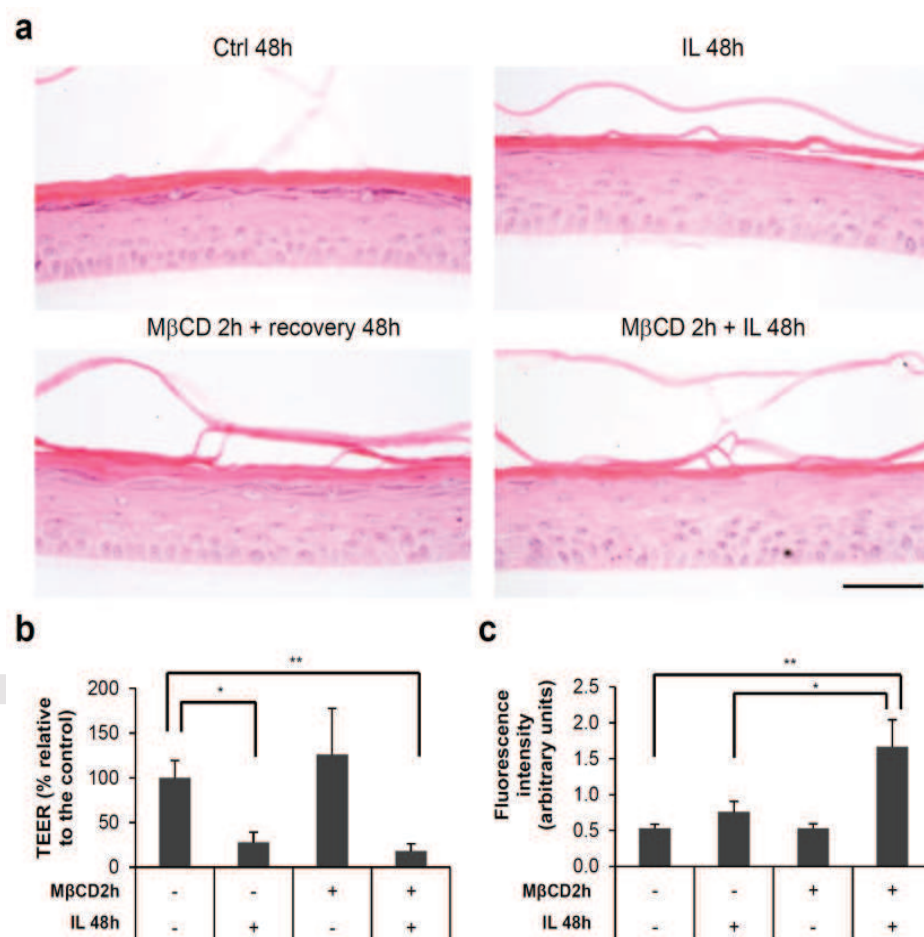
Figure 2. Transcriptional regulation of atopic dermatitis markers in treated RHE and hypothetical simplified model for AD-like pathogenesis.

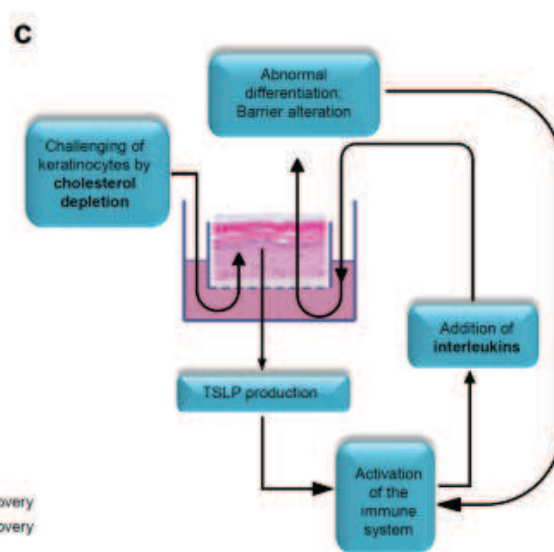
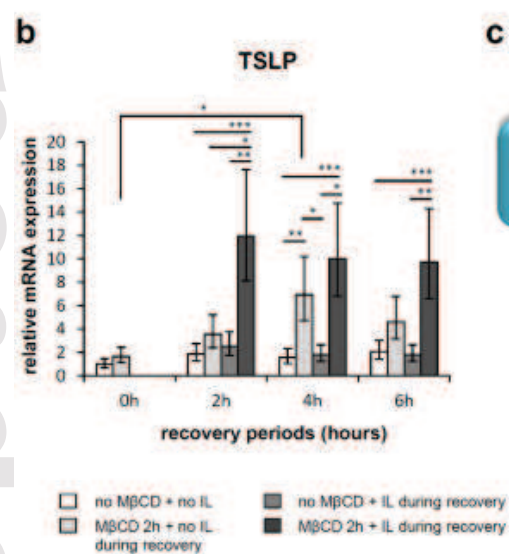
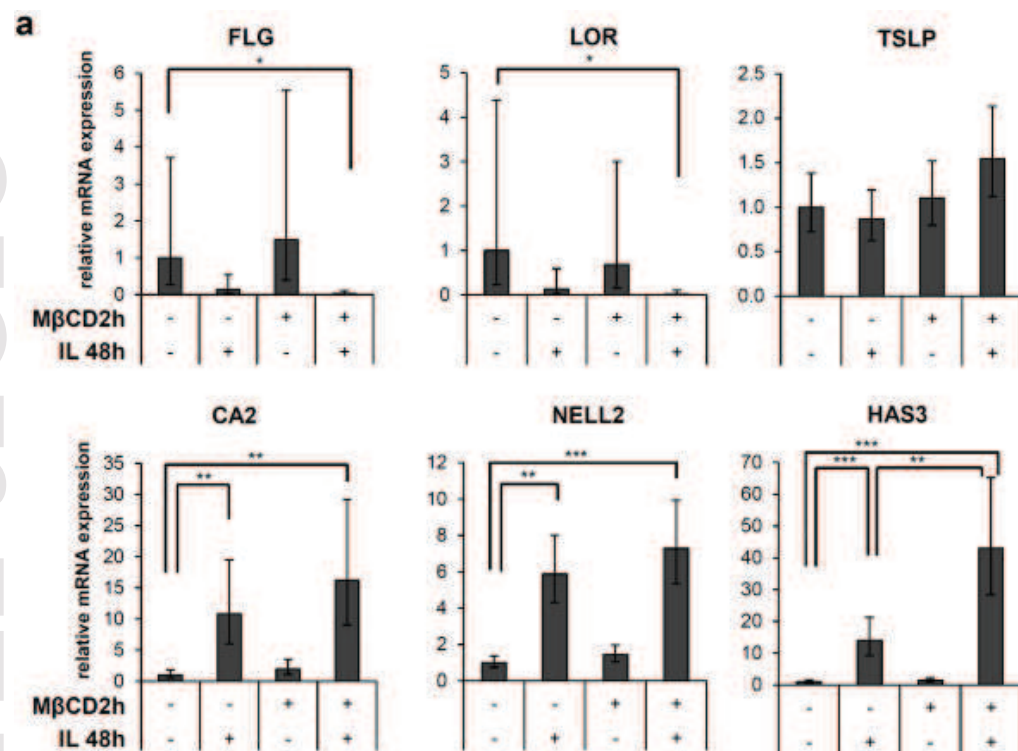
(a) RT-qPCR analysis of the expression of filaggrin (FLG), loricrin (LOR), thymic stromal lymphopoietin (TSLP), carbonic anhydrase II (CA2), neural epidermal growth factor-like 2 (NELL2) and hyaluronan synthase 3 (HAS3) were performed on total RNA extracted from RHE treated with M β CD (M β CD2h)

and/or interleukins 4, 13 and 25 (IL48h). Levels of mRNA expression in control conditions were arbitrarily fixed at 1. Error bars represent 95% confidence intervals (n=3 independent cultures, one-way RM ANOVA, *P<0.05, **P<0.01, ***P<0.001).

(b) TSLP mRNA expression was quantified by RT-qPCR in RHE incubated for 2 hours with M β CD, followed or not by incubation with IL-4, IL-13 and IL-25 for 2h, 4h or 6h. Values are expressed relative to control condition (no M β CD + no IL). Error bars represent 95% confidence intervals (n=3 independent cultures, two-way RM ANOVA, *P<0.05, **P<0.01, ***P<0.001)

(d) Plasma membrane cholesterol depletion with M β CD in RHE challenges keratinocytes which in turn produce TSLP, a Th2 response-activating cytokine. Addition of Th2-related interleukins in the culture medium intends to mimic consequences of the activation of immune cells *in vivo*. Response of challenged RHE includes morphological alterations as well as weakening of the epidermal barrier. *In vivo*, activation of the immune system resulting from altered barrier function, would maintain the vicious circle observed in AD.





Supplementary information

M β CD concurs with IL-4, IL-13 and IL-25 to induce alterations reminiscent of atopic dermatitis in reconstructed human epidermis

EVELYNE DE VUYST¹, SÉVERINE GILTAIRE¹, CATHERINE LAMBERT DE ROUVROIT¹, JEREMY MALAISSE¹, ABDALLAH MOUND¹, MAUREEN BOURTEMBOURG¹, ARJEN F. NIKKELS², ALINE CHRÉTIEN³, MICHEL SALMON³ and YVES POUMAY¹

1 Cell and Tissue Laboratory-URPHYM-Narilis, University of Namur, Namur, Belgium.

2 Department of Dermatology, University Medical Center of Liège, Liège, Belgium.

3 StratiCell, Les Isnes, Belgium.

*Correspondence to: yves.poumay@unamur.be

SUPPLEMENTARY FIGURES

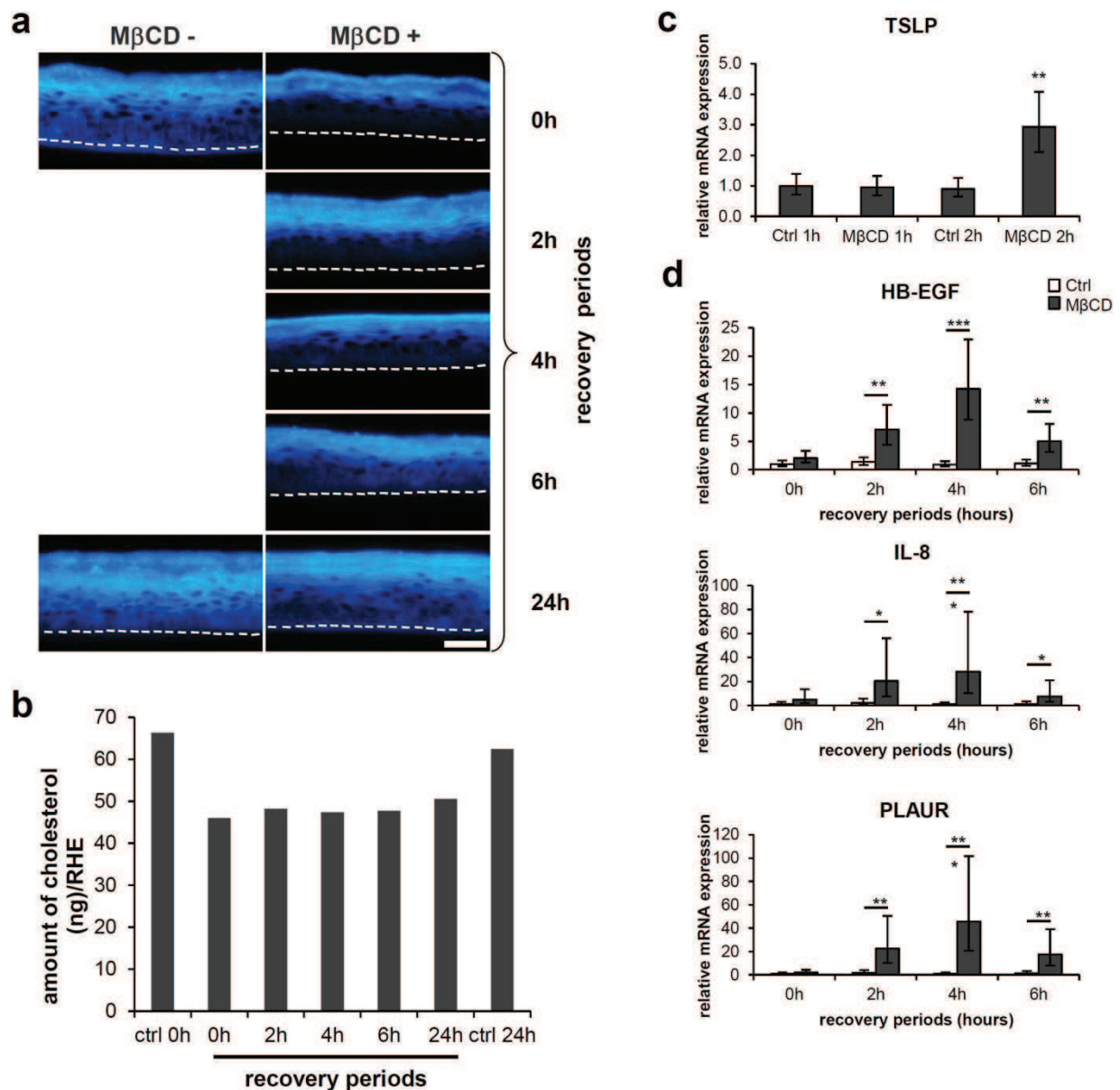


Figure S1 Effect of incubation with MβCD for 2 hours on RHE analysed at day 11 of tissue reconstruction.

(a) Localization of cholesterol. Frozen sections of RHE were stained with filipin and observed under a fluorescent microscope using UV wavelength as excitation light (DAPI filters). RHE were left untreated (MβCD -) or treated with 7.5mM MβCD for two hours (MβCD +) followed by different recovery periods (0h, 2h, 4h, 6h and 24h). White dotted lines delineate the polycarbonate filter (scale bar = 50 μm). **(b)** Cholesterol was quantified in RHE left untreated (ctrl 0h and ctrl 24h) or in RHE incubated with 7.5mM MβCD for two hours before different recovery periods (0h, 2h, 4h, 6h and 24h). **(c)** RT-qPCR analysis of TSLP expression in RHE, incubated or not with MβCD during 1h or 2h. The values are relative to the control conditions (Ctrl 1h and Ctrl 2h). **(d)** RT-qPCR analysis of heparin-binding EGF-like growth factor (HB-EGF), IL-8 and plasminogen activator urokinase receptor (PLAUR) mRNA expression levels in RHE during the recovery period after 2 hours of MβCD treatment. Error bars represent 95% confidence intervals (n=3, two-way repeated-measures analysis of variance, **P<0.01).

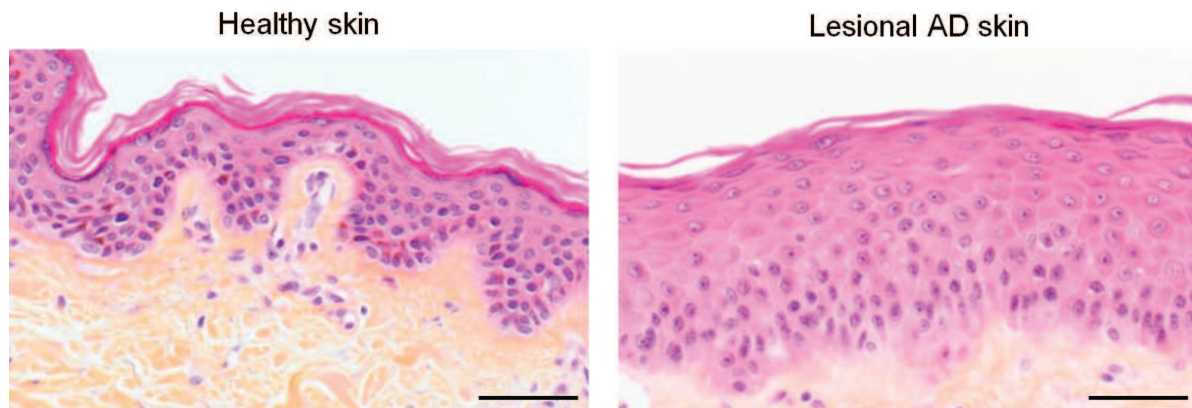


Figure S2. Morphology of healthy and lesional AD skin biopsies.

Histological analysis sections of healthy and lesional AD skin biopsies. After fixation, biopsies were embedded in paraffin before staining with haematoxylin and eosin. Scale bars=50μm.

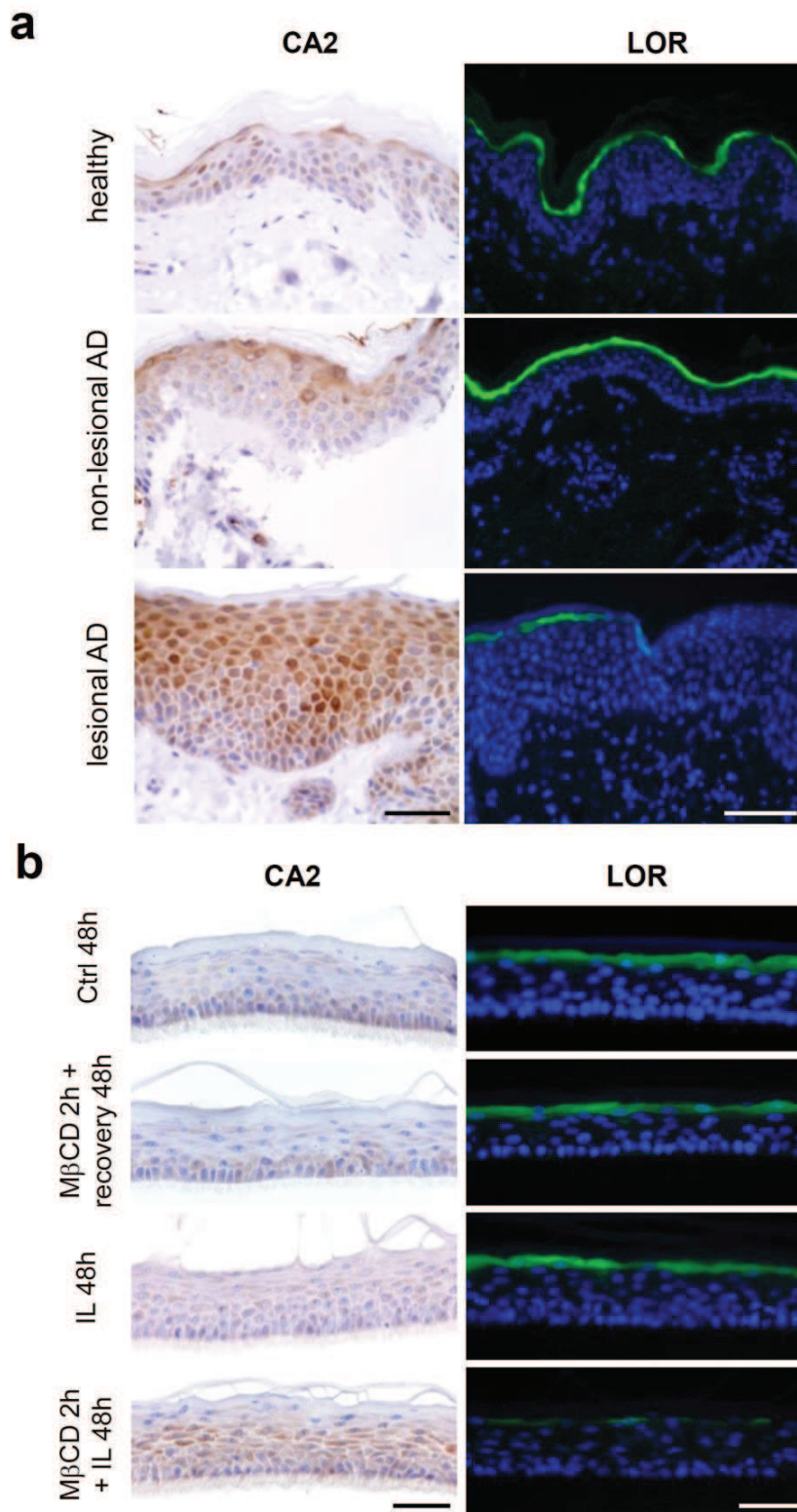


Figure S3. Labeling of atopic dermatitis-associated markers.

Immunoperoxidase-labelling of carbonic anhydrase 2 (CA2) and hematoxylin counterstaining as well as immunofluorescence labelling of loricrin (LOR) in green and nuclei in blue (Hoechst 33258).

(a) Healthy, non-lesional and lesional atopic dermatitis skin sections. (b) RHE treated for 2 hours with MβCD followed (MβCD 2h + IL 48h) or not (MβCD 2h + recovery 48 h) by 48h of incubation in the presence of IL-4, -13 and -25 (IL 48h) were processed for histology and analyzed for CA2 and LOR. Results shown are representative of three independent experiments. Scale bars= 50μm.

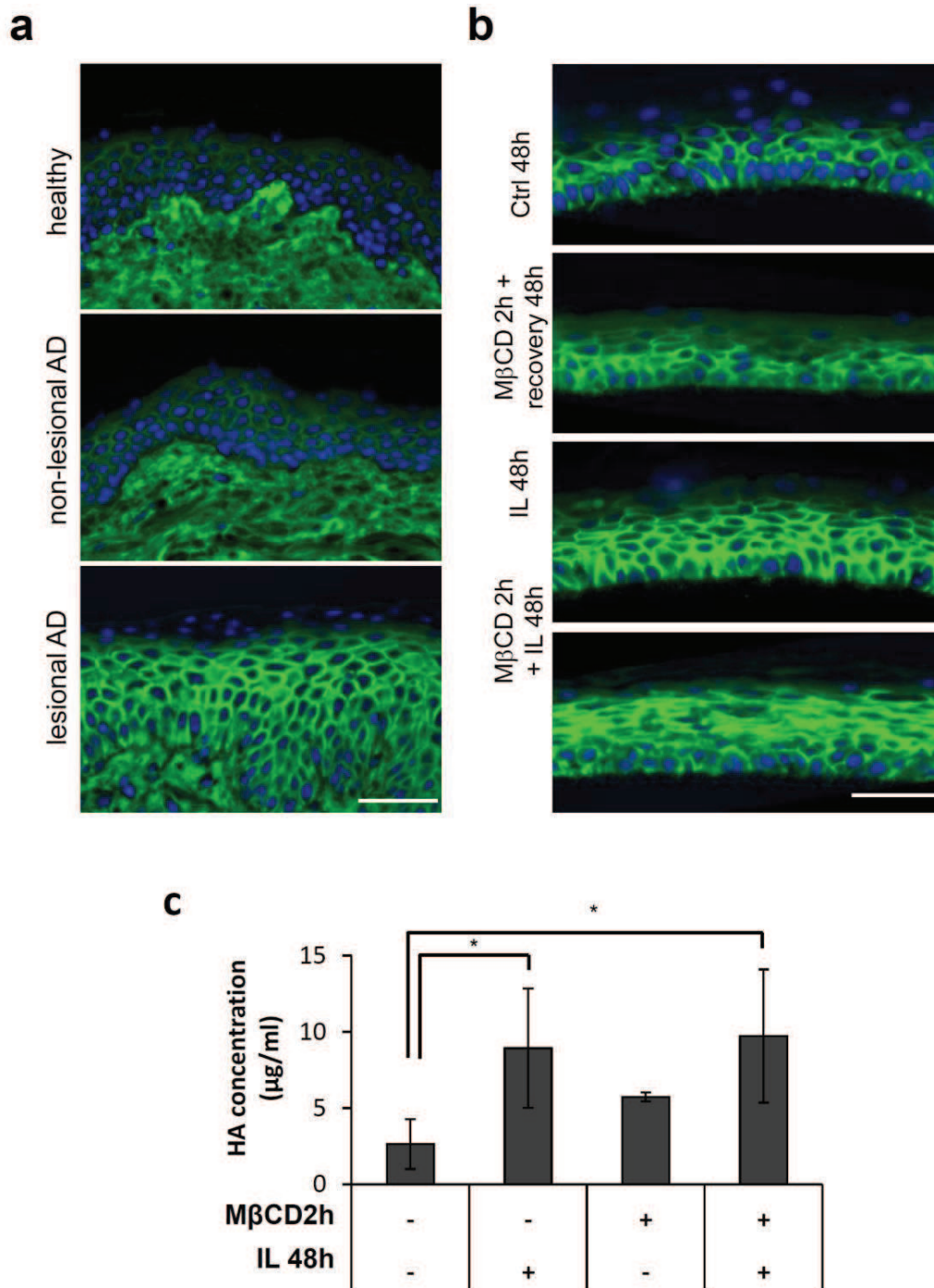


Figure S4. Hyaluronic acid (HA) staining and concentration in the culture medium.

(a) Healthy, non-lesional and lesional atopic dermatitis skin biopsies or (b) reconstructed human epidermis (RHE) were processed for histology and immunostaining of hyaluronic acid (HA) was realised using a biotinylated hyaluronic acid (HA)-binding protein staining (green). Hoechst 33258 (blue) was used for nuclei staining. RHE were treated with a combination of 2 hours of treatment with MβCD followed by 48h of interleukins 4, 13 and 25 treatment at the 11th day of reconstruction (MβCD 2h + IL 48h). These RHE were compared to RHE treated with MβCD during 2 hours followed by incubation in fresh culture medium (MβCD 2h + recovery 48 h) or treated during 48 hours with interleukins only (IL 48h) as well as untreated RHE (ctrl 48h). Results shown are representative of three independent experiments. (c) Culture media of the last 48 hours of culture were harvested and released HA concentrations were measured. Data are shown as means ± SD (n=3, one-way repeated-measures analysis of variance, *P<0.05).

MATERIALS AND METHODS

Skin biopsies and reconstruction of human epidermis

Healthy adult human skin samples were obtained from abdominoplasties (kindly provided by Dr. B. Bienfait, Clinique St. Luc, Namur-Bouge, Belgium) and keratinocytes were cultured as described (De Vuyst *et al.*, 2014; Frankart *et al.*, 2012; Poumay *et al.*, 2004). After 11 days of culture, RHE were treated for 2 hours with 7.5 mM M β CD (Sigma-Aldrich, Diegem, Belgium). A treatment for two hours with M β CD was selected because it induced a significant increase in expression of thymic stromal lymphopoietin (TSLP), a well-known actor in AD (Figure S1c), without alteration of RHE viability (data not shown). Transient extraction of cholesterol as well as activation of known target genes by M β CD was evaluated (Figure S1) confirming previous data (De Vuyst *et al.*, 2015).

After M β CD treatment, RHE were rinsed before culture for the next 48 hours in the presence of respectively 50 ng/ml IL-4, 50 ng/ml IL-13, and 20 ng/ml IL-25 (Peprotech, London, UK). The choice for IL-4 and IL-13 was first based on experiments already published showing elevated expression levels of these cytokines in lesional AD skin (Howell *et al.*, 2009; Tazawa *et al.*, 2004). Further interesting, these interleukins could induce a significant decrease of filaggrin expression in primary human keratinocytes (Howell *et al.*, 2009; Pellerin *et al.*, 2013). Furthermore, other in vitro models of AD also used cytokine mix and notably IL-4 and IL-13 (Bernard *et al.*, 2012; Danso *et al.*, 2014; Kamsteeg *et al.*, 2011). IL-25 was added for different reasons. First, because increased levels of this cytokine and its receptor were detected in the epidermis of AD patients (Aktar *et al.*, 2015; Hvid *et al.*, 2011; Wang *et al.*, 2007). Besides this reason, IL-25 would play a dual role in AD, promoting type 2 cell immunity (Salimi *et al.*, 2013; Wang *et al.*, 2007), but also inhibiting filaggrin (FLG) expression in keratinocytes (Hvid *et al.*, 2011; Pellerin *et al.*, 2013), thereby affecting skin barrier in AD patients. Therefore, acting both on immune and epidermal cells, IL-25 may provide, in collaboration with TSLP, some explanation for the link observed between inflammation and barrier disruption in AD lesions (Deleuran *et al.*, 2012). Furthermore, in the skin of AD patients, expression of IL-25 and its receptor was increased after exposure to allergens (Corrigan *et al.*, 2011). For all these reasons, we thought it could be of interest as a potentially therapeutic target and could therefore be added in our model contribute to the development of the pathogenesis, and partly explain the vicious circle observed in AD.

AD biopsies were collected from lesional or surrounding non-lesional skin of patients suffering from longstanding disease.

This study was conducted according to the Declaration of Helsinki Principles and approved by the Ethics Committees.

Morphological analysis and labelling

RHE or biopsies were embedded in paraffin. Sections were stained with haematoxylin-eosin (safran). Images were obtained by light microscopy using Olympus BX63 microscope.

For CA2 immunodetection, antigen retrieval was performed by heat-induction. Anti-rabbit CA2 from Sigma-Aldrich (Diegem, Belgium) was diluted 1:500, anti-rabbit LOR from Abcam (Cambridge, UK) 1:100 and biotinylated HA-binding complex (generously provided by Prof. M. Tammi) was diluted 1:50. Tissue samples were incubated for one hour with anti-rabbit HRP secondary antibody (Vectastain ABC kit, Vector Laboratories, Peterborough, UK) diluted 1:100 for the CAII staining or with Alexa 488 conjugated anti-rabbit IgG (Life technologies, New York, USA) also diluted 1:100 for the LOR staining or with fluorescein-labelled streptavidin (Vector Laboratories, Peterborough, UK) for the HA labelling.

TEER measurements and permeability to lucifer yellow

TEER measurements were performed using Millicel electrical resistance system from Merck-Millipore (Overijse, Belgium). The fluorescent dye lucifer yellow was obtained from Sigma-Aldrich (Diegem, Belgium) and applied topically at a concentration of 1 mM in PBS on the RHE for 6 hours at 37°C. Afterwards, the medium was collected for fluorescence measurement (λ_{ex} : 485nm; λ_{em} : 535nm).

Analysis of mRNA expression by RT-qPCR

Total RNA was extracted from RHE using RNeasy kit (Qiagen, Hilden, Germany), reverse transcribed using the Super Script II RNase H⁻reverse transcriptase kit (Invitrogen, Merelbeke, Belgium) and amplified with the FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) in the Light Cycler® 96 Real-Time PCR system (Roche, Diagnostics, Mannheim, Germany). Results were normalized to the RPLP0 reference gene (Minner and Poumay, 2009; Mound *et al.*, 2015). Primers (Eurogentec, Liège, Belgium) were used at a concentration of 300 nM. Sequences are available in table S1. Data were analyzed using the $\Delta\Delta\text{Cq}$ quantitative method and 95% confidence intervals were calculated after one-way RM ANOVA followed by Tukey tests or two-way RM ANOVA followed by Bonferroni correction using SigmaStat 3.0.

TABLE S1: Oligonucleotides used to perform the RT-qPCR

	Forward (5' – 3')	Reverse (5' – 3')
RPLP0	ATCAACGGGTACAAACGAGTC	CAGATGGATCAGCCAAGAAGG
FLG	GGGCACTGAAAGGCAAAAAG	CACCATAATCATAATCTGCACTACCA
LOR	TCATGATGCTACCCGAGGTTTG	CAGAACTAGATGCAGCCGGAGA
HAS3	GCCCTCGGCGATTCTG	TGGATCCAGCACAGTGTCTAGA
CAII	AACAATGGTCATGCTTTCAACG	TGTCCATCAAGTGAACCCAG
NELL2	TAAGGGTATAATGCAAGATGTCCAATT	AGATCTGGGCACTGAGCAATAAA
TSLP	CGCGTCGCTCGCCAAAGAAA	TGAAGCGACGCCACAATCCTTG
HAS1	CCTCACCAACCGCATGCT	GGACGAGGGCGTCTCTGA
HB-EGF	TGGCCCTCCACTCCTCATC	GGGTCACAGAACCATCCTAGC

Filipin staining

Epidermis were embedded in OCT (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and cryopreserved at -80°C. Frozen sections of RHE were realised and fixed for 30 min with 4% paraformaldehyde at room temperature. After a washing step in PBS, sections were incubated with 50 µg/ml of filipin from Sigma-Aldrich (Diegem, Belgium) for 30 min at room temperature. After a washing step, sections were mounted using Mowiol (Molecular Probes, Ghent, Belgium) and filipin was visualised by epifluorescence using Olympus AX70 microscope with a UV filter. Pictures were taken using uniform conditions (quickest exposure times to avoid the rapid bleaching of filipin).

Cholesterol assay

Cellular cholesterol was extracted and purified from RHE by a procedure derived from the protocol of Selvais *et al.* (Selvais *et al.*, 2011). After lysis of RHE in chloroform, washing steps were realised using successively methanol, NaCl and CaCl₂/methanol. After centrifugation, the organic phase was collected, Triton X-100/acetone was added and samples were evaporated before being solubilised in demineralised water. Cholesterol was assayed using the Amplex Red Cholesterol Assay kit from Molecular probes (Ghent, Belgium) according to the manufacturer's protocol.

HA quantification

HA was quantified in culture media using Hyaluronan DuoSet kit (R&D systems, Abingdon, UK).

Aktar MK, Kido-Nakahara M, Furue M, *et al.* (2015) Mutual upregulation of endothelin-1 and IL-25 in atopic dermatitis. *Allergy* 70:846-54.

Bernard FX, Morel F, Camus M, *et al.* (2012) Keratinocytes under Fire of Proinflammatory Cytokines: Bona Fide Innate Immune Cells Involved in the Physiopathology of Chronic Atopic Dermatitis and Psoriasis. *Journal of allergy* 2012:718725.

Corrigan CJ, Wang W, Meng Q, *et al.* (2011) Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses. *The Journal of allergy and clinical immunology* 128:116-24.

Danso MO, van Drongelen V, Mulder A, *et al.* (2014) TNF-alpha and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *J Invest Dermatol* 134:1941-50.

De Vuyst E, Charlier C, Giltair S, *et al.* (2014) Reconstruction of normal and pathological human epidermis on polycarbonate filter. *Methods Mol Biol* 1195:191-201.

De Vuyst E, Giltair S, Lambert de Rouvroit C, *et al.* (2015) The activation of cultured keratinocytes by cholesterol depletion during reconstruction of a human epidermis is reminiscent of monolayer cultures. *Arch Dermatol Res* 307:309-18.

Deleuran M, Hvid M, Kemp K, *et al.* (2012) IL-25 induces both inflammation and skin barrier dysfunction in atopic dermatitis. *Chem Immunol Allergy* 96:45-9.

Frankart A, Malaisse J, De Vuyst E, *et al.* (2012) Epidermal morphogenesis during progressive in vitro 3D reconstruction at the air-liquid interface. *Exp Dermatol* 21:871-5.

Howell MD, Kim BE, Gao P, *et al.* (2009) Cytokine modulation of atopic dermatitis filaggrin skin expression. *The Journal of allergy and clinical immunology* 124:R7-R12.

Hvid M, Vestergaard C, Kemp K, *et al.* (2011) IL-25 in atopic dermatitis: a possible link between inflammation and skin barrier dysfunction? *J Invest Dermatol* 131:150-7.

Kamsteeg M, Bergers M, de Boer R, *et al.* (2011) Type 2 helper T-cell cytokines induce morphologic and molecular characteristics of atopic dermatitis in human skin equivalent. *Am J Pathol* 178:2091-9.

Minner F, Poumay Y (2009) Candidate housekeeping genes require evaluation before their selection for studies of human epidermal keratinocytes. *J Invest Dermatol* 129:770-3.

Mound A, Malaisse J, De Vuyst E, *et al.* (2015) Epidermal reference genes at the forefront of data interpretation. *Exp Dermatol* 24:738-9.

Pellerin L, Henry J, Hsu CY, *et al.* (2013) Defects of filaggrin-like proteins in both lesional and nonlesional atopic skin. *The Journal of allergy and clinical immunology* 131:1094-102.

Poumay Y, Dupont F, Marcoux S, *et al.* (2004) A simple reconstructed human epidermis: preparation of the culture model and utilization in in vitro studies. *Arch Dermatol Res* 296:203-11.

Salimi M, Barlow JL, Saunders SP, *et al.* (2013) A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *The Journal of experimental medicine* 210:2939-50.

Selvais C, D'Auria L, Tyteca D, *et al.* (2011) Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1 (LRP-1) and clearance function. *FASEB J* 25:2770-81.

Tazawa T, Sugiura H, Sugiura Y, *et al.* (2004) Relative importance of IL-4 and IL-13 in lesional skin of atopic dermatitis. *Arch Dermatol Res* 295:459-64.

Wang YH, Angkasekwinai P, Lu N, *et al.* (2007) IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *The Journal of experimental medicine* 204:1837-47.